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Legionella pneumophila Philadelphia-1 *tatB* and *tatC* affect intracellular replication and biofilm formation

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Abstract

Legionella pneumophila is a facultative intracellular human pathogen and an important cause of Legionnaires' disease, a severe form of pneumonia. Recently, we showed the presence of a putative twin-arginine translocation (Tat) pathway in *L. pneumophila* Philadelphia-1. This secretion pathway is used to transport completely folded proteins across the cytoplasmic membrane. The importance of the Tat pathway in *L. pneumophila* was investigated by constructing a *tatB* and a *tatC* mutant. Functionality of the Tat pathway was shown using a proven heterologous Tat substrate. It was shown that *tatB* and *tatC* are involved in intracellular replication in *Acanthamoeba castellanii* and differentiated U937 cells, and in biofilm forming ability. A putative *Legionella* Tat substrate was identified via 2D gel electrophoresis.

Keywords: Twin arginine; Tat secretion; *Legionella pneumophila*; Virulence; Biofilms; Signal peptides

Legionella pneumophila is a Gram-negative, facultative intracellular pathogen, causing an often fatal form of pneumonia, better known as Legionnaires' disease. In the environment, this bacterium multiplies primarily within protozoa and can be found within biofilms. Upon infection, by inhalation of contaminated aerosols, replication takes place in human alveolar macrophages [1].

Different protein secretion pathways have been characterised as important determinants of virulence. For the translocation of proteins across the inner and outer membrane, a type II, two type IV, and a putative type I secretion system have been described for *L. pneumophila* (see review [2]). In addition to these secretion systems, we identified the presence of a putative twin-arginine translocation pathway (Tat pathway) [3]. The latter pathway functions in parallel with the Sec pathway for translocation of proteins across the cytoplasmic membrane. Typical for the Tat secretion system is that it delivers folded proteins into the periplasm or into an outer membrane secretion system, using the proton motive force of the cell as energy source. Tat-dependent precursor proteins carry in their signal peptide two arginine residues, embedded in the conserved motif (S/T)-R-R-X-F-L-K, although naturally occurring Tat substrates with a lysine–arginine dyad [4] and a RNR motif [5] have also been reported. It has been shown that the Tat machinery can be a species-specific system, not allowing translocation of heterologous Tat substrates [6].

The bacterial Tat pathway is best studied in *Escherichia coli*. In *E. coli*, the Tat apparatus is encoded by the *tatA*, *tatB*, *tatC*, and *tatE* gene. *TatA*, *tatB*, and *tatC* are constituting an operon, while *tatE* is located elsewhere on the chromosome [7]. In *L. pneumophila*, genes encoding *E. coli* TatA, TatB, and TatC homologues were identified. Here the *tatA* and *tatB*

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gene are cotranscribed from the same promoter, while *tatC* is monocistronic and located distantly from *tatAB*. It was shown that the three *tat* genes are also expressed intracellularly [3].

It became clear that the Tat pathway plays an important role in bacterial virulence in general. In the opportunistic human pathogen *Pseudomonas aeruginosa*, two phospholipases C are secreted via the Tat pathway [8]. The *P. aeruginosa tatC* mutant also shows decreased lesion of lung tissue in an animal model [9]. In the enterohemorrhagic *E. coli* O157:H7 strain, the Tat pathway is necessary for the secretion of the Shiga toxin and a *tatC* mutant was shown to be less cytotoxic for Vero cells [10]. In case of the plant pathogen *Agrobacterium tumefaciens*, the *tatC* mutant failed to induce tumor formation [11]. For each of these pathogens, the Tat system was also reported to contribute to motility of the bacteria [9–11]. Furthermore, the Tat pathway has been shown to be involved in biofilm formation for *P. aeruginosa* [9] and chemotaxis for *E. coli* O157:H7 [10].

In parallel with our present study, the role of the *tatB* gene of *L. pneumophila* 130b in phospholipase C secretion, growth under iron-limiting conditions, and intracellular replication was very recently investigated [12]. In the current study, we analysed the role of the Tat pathway in *L. pneumophila* Philadelphia-1 by insertional inactivation of the *tatB* and *tatC* gene. Independent inactivation of both genes encoding proteins with a totally different function in Tat-dependent translocation, with TatC being generally considered as the most important component, will allow us to investigate if similar phenotypes are generated. In addition, as in silico analysis shows that predicted Tat substrates differ for several *L. pneumophila* strains [12], one may assume a different role for the Tat pathway in different strains. In this study, functionality of the *L. pneumophila* Philadelphia-1 Tat pathway was proven by using a heterologous established Tat substrate. Furthermore, it was demonstrated that the *tatB* and the *tatC* mutant both are impaired for replication in the amoeba *Acanthamoeba castellanii* and in differentiated U937 cells. In addition, we showed that the Tat pathway in *L. pneumophila* plays a role in biofilm forming ability.

Materials and methods

Bacterial strains, plasmids, and growth conditions. *Legionella pneumophila* ATCC33152 and derivatives thereof were grown at 37 °C on buffered charcoal yeast extract (BCYE) agar plates or in buffered yeast extract broth (BYE) supplemented with α-ketoglutarate, L-cysteine, and ferric pyrophosphate [13]. Where necessary, kanamycin (10 μg ml^{-1}) or chloramphenicol (5–12 μg ml^{-1}) was added. For counterselection, based on the *sacB* gene, 7% sucrose was added to the solid medium. *E. coli* TG1 was used as a host for cloning purposes [14]. Cultures were grown in Luria–Bertani medium [15], if necessary supplemented with ampicillin (50 μg ml^{-1}), kanamycin (50 μg ml^{-1}) or chloramphenicol (25 μg ml^{-1}). The plasmids used in this study are summarized in Table 1.

For monitoring growth, 50 ml of medium was inoculated with an overnight culture to an OD_{600} of 0.1 and samples (1 ml) were taken every 2 h and cell density was measured at OD_{600} in a Bio-Rad SmartSpec 3000 spectrophotometer.

For quantification of biofilm formation an overnight culture was diluted to an OD_{600} of 0.4 in BYE medium in a polystyrene 24-well plate (Greiner). Plates (1 ml of culture per well) were incubated without shaking at 37 °C during 72 h. Then the cell density was measured, non-adherent cells were removed, and cells attached to the polystyrene were stained with 0.1% crystal violet. After extensive washing with distilled water, 1 ml ethanol/acetone (80:20) was added. Biofilm for-

Table 1
Plasmids used in this study

Plasmid	Characteristics	Source or reference
pGEM-T Easy	Used for cloning of PCR amplified DNA fragments, Ap^R	Promega
pBluescriptIIKS(+)	Ap^R, *lacZα*, MCS	Stratagene
pBCKS(+)	Cm^R, *lacZα*, MCS	Stratagene
pBCN	pBCKS(+) derivative with an introduced *Nde*I restriction site downstream the *lac* promoter	This work
pBOC20	*E. coli–Legionella* shuttle vector, Cm^R, counterselection based on *sacB* gene	[18]
pBSKan	pBluescriptIIKS(+) derivative with the *neo* gene from pFD666 cloned as *Eco*RI restriction fragment	[17]
pGEMtatAB	pGEM-T Easy derivative with a cloned 733 bp PCR fragment containing the *L. pneumophila tatA* and *tatB* gene	This work
pGEMABKan	pGEM-T Easy derivative containing the *L. pneumophila tatA* and *tatB* gene with the *neo* gene cloned into the *tatB* gene	This work
pBOC20ABKan	pBOC20 derivative containing the insert of pGEMABKan	This work
pBCtatAB	pBCKS(+) derivative containing the *L. pneumophila tatA* and *tatB* gene	This work
pBSUKD	pBluescriptIIKS(+) derivative containing the *tatC* upstream and downstream flanking sequences at both sides of the *neo* gene	This work
pBOCUKD	pBOC20 derivative containing the insert of pBSUKD	This work
pBCtatC	pBCKS(+) derivative containing the *L. pneumophila tatC* gene	This work
pTAP	*T. thermophilus* HB27 *tap* gene in pBAD24	[19]

Ap^R, ampicillin resistance; Cm^R, chloramphenicol resistance; and MCS, multiple cloning site.